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## A. INTRODUCTION

Genetic alterations at the short arm of chromosome 11 are a frequent event in the etiology of cancer. Several childhood tumors demonstrate LOH for 11p including rhabdomyosarcoma (1), adrenocortical carcinoma (2), hepatoblastoma (3), mesoblastic nephroma (4) and Wilms' tumors (5). Recurrent LOH at 11p is also observed in adult tumors including bladder (6), ovarian (7), lung carcinomas (8), testicular cancers (9), hepatocellular carcinomas (10) and breast carcinomas (11,12), suggesting the presence of one or more critical tumor gene(s) involved in several malignancies. We have identified loss of heterozygosity (LOH) of 11p15 and microsatellite instability at a specific marker D11S988 on chromosome 11p15 as late genetic events in mammary tumorigenesis (11). This suggests a crucial role for this region in breast cancer progression. More recently, we have mapped and identified two distinct regions on chromosome 11p15 that are subject to LOH during breast tumor progression and metastasis (12). We have found a significant correlation between loss of heterozygosity at the two chromosomal regions and the clinical and pathological features of the breast tumors. LOH in region 1 correlated with tumors that contain ductal carcinoma *in situ* synchronous with invasive carcinoma. This suggests that the loss of a critical gene in this region may be responsible for early events in malignancy or invasiveness. LOH at region 2 correlated with clinical parameters which portend a more aggressive tumor and a more ominous outlook for the patient, such as aneuploidy, high S-phase fraction and the presence of metastasis in regional lymph nodes. Our data strongly suggests the presence of a metastasis suppressor gene on chromosome 11p15.5 (12). Winquist et al (13) have shown that LOH for chromosome 11p15 is associated with poor survival after metastasis. The association between 11p LOH, tumor progression and metastasis, that we describe, is analogous to the observations made in other epithelial tumors. For example, LOH at 11p correlated with advanced T stage and nodal involvement in Non-small cell lung carcinoma (14) as well as subclonal progression, hepatic involvement (15), and poor survival in ovarian and breast carcinomas (7,13). Phillips et. al. (16) have shown that micro-cell mediated transfer of a normal human chromosome 11 into the highly metastatic breast cancer cell line MDA-MB-435, had no effect on tumorigenicity in nude mice, but suppressed metastasis to the lung and regional lymph nodes. These studies further support our observation that chromosome 11 harbors a metastasis suppressor gene.

An intriguing candidate gene that we have mapped to the metastasis suppressor locus on chromosome 11p15.5 is the Integrin-linked kinase (ILK)(12). ILK is a newly identified ankyrin-repeat containing serine/threonine kinase (17), that binds to the cytoplasmic domains of both  $\beta 1$  and the  $\beta 3$  integrins. The kinase activity of ILK is modulated by interaction of cells with components of the extracellular matrix or by integrin clustering (17,18). Interactions between integrins and their ligands are involved in the regulation of many cellular functions, including embryonic development, cell proliferation, tumor growth and the ability to metastasize (19). Integrins are composed of non-covalently associated  $\alpha$ - and  $\beta$ - glycoprotein sub-units, and receptor diversity and ligand specificity is generated by the various associations of at least eight known  $\beta$ -subunits and 14  $\alpha$ -subunits (20). The majority of integrins are receptors for ECM proteins such as collagens ( $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$ ,  $\alpha 3\beta 1$ ), fibronectin ( $\alpha 3\beta 1$ ,  $\alpha 4\beta 1$ ,  $\alpha 5\beta 1$ ), laminin ( $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$ ) or vitronectin ( $\alpha v\beta 3$ ,  $\alpha v\beta 5$ ). In recent years, it has become apparent that integrins do not function merely as transmembrane rivets, linking the cell to the ECM, but they are also involved in signaling (21). Integrins signal into the cell, as conventional receptors, but, in addition, are also able to transmit information from within the cell to the matrix as well as to other cells via a mechanism termed inside-out signaling (22). The precise mechanisms involved in integrin-mediated signaling are unknown but it appears that they may involve G-proteins, tyrosine kinases, and serine/threonine kinases including ILK (23).

The role of Integrin-linked kinase in integrin-mediated signaling is not fully understood. The activation or inhibition of ILK is cell-type dependent and can be modified by growth factors (24). Previous studies (18,24) have reported two contrasting effects of

ILK on the cell. Overexpression of ILK in rat intestinal epithelial cells results in the stimulation of anchorage-independent cell growth (24), and cell cycle progression caused by the constitutive up-regulation of cyclin D1 and cyclin A, resulting in the hyperphosphorylation of the retinoblastoma protein (24). Overexpression of ILK in rat epithelial cells also results in the induction of tumorigenicity in nude mice indicating that ILK is a protooncogene (25). Surprisingly however, transient or stable expression of ILK in epithelial cells results in rapid stimulation of fibronectin matrix assembly (26). Fibronectin (Fn) is a major constituent of extracellular matrices deposited during embryogenesis and wound-healing (27). The assembly of Fn matrix is a highly regulated cellular process in which soluble, dimeric Fn molecules are assembled into an insoluble, fibrillar pericellular matrix (28). A common feature of many oncogenically transformed cells is that they lose the ability of assembling a Fn matrix (29). In contrast, oncogenic transformation frequently induces anchorage-independent growth, *in vitro*, and is a specific correlate of tumor growth *in vivo* (25). Thus, it is likely that these divergent and paradoxical effects mediated by ILK may depend on the particular cell-type and the cell-specific integrins that are activated in a cell.

## **B. BODY:**

### **B1. Mapping of ILK to the tumor suppressor locus (LOH region 2) on 11p15.5:**

To build a foundation for the isolation of genes in 11p15.5, we constructed a 500 kb genomic contig that includes the critical region between D11S1338 and D11S1323. The genomic contig (Figure 1) consisting of PAC, BAC, YAC and cosmid clones encompass the entire region spanning the breast tumor-suppressor gene, the location of which was defined by LOH analysis. We mapped seven novel transcripts and three previously reported genes to the critical region between D11S1338 and D11S1323 and the remaining seven transcripts just proximal to D11S1323. The known genes TAF II 30 (Tata box-binding protein-associated protein), Lysosomal pepstatin insensitive protease (CLN2) and Integrin -linked kinase (ILK) were previously mapped only at the level of cytogenetic resolution. However, as shown in Fig. 1, we have been able to determine the precise genomic locations of these three genes. The map location and its role in integrin-mediated signal transduction makes ILK an attractive candidate metastasis suppressor gene. The following preliminary studies were conducted to evaluate the possible role of ILK in breast tumorigenesis: 1) Expression patterns of ILK in normal breast /breast tumors and in breast cancer cell lines. 2) Effects of ILK overexpression in the metastatic breast cancer cell line MDA-MB-435.

### **B2. Expression patterns of ILK.**

**D1a. mRNA expression:** A partial cDNA isolated by cDNA selection was used to obtain a full length clone from a human placental cDNA library and its identity confirmed by sequence analysis and database comparison. Northern blot hybridization was done to compare the mRNA expression in normal breast versus tumor tissue. As shown in Figure 2a, ILK mRNA is highly expressed in normal breast tissue and is considerably down-regulated in primary breast tumors. Comparison of ILK mRNA expression in different breast cancer cell lines (Figure-2b) revealed that ILK mRNA is expressed in the non-metastatic breast cancer cell lines MCF-7, T47D, ZR75.1 and MDA-134, the expression is down-regulated in the metastatic breast cancer cell lines MDA-MB-435 and MDA-MB-231. These results suggest that ILK mRNA expression correlates inversely with tumorigenicity and/or metastatic potential.

**B2b. ILK expression in human breast tissue specimens:** ILK expression was examined using indirect immunofluorescence microscopy in normal human breast tissue and in corresponding primary breast tumors. A total of 20 frozen samples of 10 normal breast tissues and 10 infiltrating ductal carcinomas were used for indirect immunostaining. ILK specific antibody was purchased from Upstate Biotechnology Inc. All the immunohistochemical determinations were performed on representative samples snap-

frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until sectioning. Cryostat sections, 4-6  $\mu\text{m}$  thick, were fixed in cold acetone at  $4^{\circ}\text{C}$  for 10 min., air-dried and incubated at room-temperature with the above antibodies for 1 h. After being washed with PBS, bound antibodies were visualized using rhodamine conjugated anti-rabbit IgG secondary antibody. Sections were visualized by fluorescence microscopy. The optimal concentration of primary and secondary antibody was determined by titration and ranged from 1:50-1:200. For negative controls, in all instances, we used nonspecific IgG as the primary antibody.

As shown in Figure 3, staining of normal breast tissue with ILK-specific primary antibody and rhodamine labeled secondary antibody shows specific staining of the epithelial cells surrounding the lumen (3N). ILK expression is particularly intense in epithelial cells both within large ducts and within terminal duct lobular units but not in the stromal compartment. Incubation with purified nonspecific rabbit immunoglobulin IgG, did not result in any positive staining of the normal epithelium of the breast (control). The normal breast tissue from four representative patients were positive, (3N, 12N, 6N and 10N) whereas very low expression or undetectable ILK expression was seen in the four corresponding infiltrating ductal carcinomas (3T, 12T, 6T, 10T). These findings suggest a biological role for ILK in normal breast tissue and a potentially pivotal alteration in ILK expression during the progression of breast cancer. That ILK was expressed predominantly in normal breast tissue but was not detected in different breast cancers suggests its potential prognostic value in the etiology of breast cancer.

### **B3. Effects of overexpression of ILK in the metastatic breast cancer cell line MDA-MB-435.**

To assess the impact of overexpression of ILK on the malignant phenotype of the established human breast cancer cell line, MDA-MB-435, the human mammary cancer cells were transfected with a mammalian expression vector pIRES-EGFP (Clontech Labs.) containing ILK full length cDNA under control of the CMV promoter. We transfected near confluent MDA-MB-435 cells with either vector alone (control) or pIRES-EGFP containing ILK. The cells were transfected with 8 $\mu\text{g}$  of either plasmid using a liposome-based transfection method (30). Forty eight hours after transfection, we sorted the cell culture using a FACScan (Becton-Dickison) flow cytometer equipped with an argon laser emitting at 488nm. To obtain stable transfectants, the transiently transfected cells sorted by flow cytometry and cultured in complete medium containing 600  $\mu\text{g}/\text{ml}$  G418 (GIBCO-BRL). Stable cell lines were obtained 3-4 weeks after G418 selection. A total of six MDA-MB-435 stable clones expressing ILK have been established. Four control stable transfectants have been also obtained.

#### B3a. ILK protein expression in transfected and control cells:

Stable transfectants of MDA-MB-435 were analyzed for ILK protein expression. ILK protein levels in transfected and untransfected cells was determined by indirect immunofluorescence as described earlier for normal and breast tumor tissue (Section B2b). High levels of ILK are expressed by the transfected MDA-MB-435 cells (Figure 4c) compared to the untransfected control (Figure-4b). The ILK protein is localized in the cytoplasm with intense perinuclear staining. Controls included omission of the primary antibody (Figure 4a). Our protein expression data suggests that we have successfully established stable transfectants of MDA-MB-435 cells overexpressing ILK.

#### B3b. Growth inhibition by ILK:

Stable transfectants or control cells ( $10^4$  cells each) were plated in 35-mm tissue culture dishes in triplicate and incubated at  $37^{\circ}\text{C}$  for 7 days. Everyday, one set of each culture dishes was trypsinized, and the cell numbers per dish were measured by Coulter counting and also using a hemocytometer. Thus, we examined the overexpression effects of ILK on the growth -rate of ILK transfectants with that of cells transfected with vector alone. As shown in Figure-5a, the initial clonal growth rate of the ILK transfectants was



several times slower than that of cells transfected with vector alone or untransfected cells. Overexpression of ILK cDNA causes the overexpressors to grow to a lower saturation density (Figure-5a) and there is substantial growth inhibition of confluent cells, as seen by the 40-45% decrease in growth rate of transfected cells compared to control cells. This growth suppression was identical in three independent experiments. One of the effects of ILK overexpression in the MDA-MB-435 cells is that transfected cells grow at a lower saturation density than control cells. Expression of ILK in MDA-MB-435 results in an accumulation of cells in the G0/G1 phase of the cell cycle, suggesting arrest predominantly at the G1/S boundary (Fig.5b). There was no evidence of apoptosis as determined by propidium staining (data not shown). The vector alone did not show the effects of ILK.

**B3c. Inhibition of tumor cell growth *in vivo*:**

The mammary carcinoma cell line MDA-MB-435 forms tumors at the site of orthotopic injection and metastasizes in nude mice. To investigate whether ILK expression affected tumor formation in nude mice, ILK transfectants and matched controls were inoculated into the subaxillary mammary fat pads of 4-6 week old athymic nude mice. Tumors were measured weekly thereafter to assess the growth rate. When  $5 \times 10^5$  cells of ILK-transfectant clone 5 or a vector transfectants were injected s.c. into the mammary gland area of mice, 8 of 8 vector-transfected mice showed palpable tumors within 8-10 days of injection, and these grew progressively, reaching a  $2.0 \times 2.0 \text{ cm}^2$  size by 12 weeks (Fig. 6a and b). As expected, histopathology of one of these tumors demonstrated that the tumor was an adenocarcinoma (data not shown). In contrast to vector-transfected MDAMB-435 cells, only two of eight mice implanted with ILK-transfected MDA-MB-435 showed any palpable or visible tumors by 12 weeks. These tumors were very small ( $<0.25 \times 0.25 \text{ cm}^2$ ) by 12 weeks. No tumors were seen in lung or lymph nodes from mice injected with ILK transfectants, whereas vector controls produced neoplastic pleural effusions and multifocal, pulmonary parenchymal metastatic carcinoma. Additional tumor masses were present in central venous blood vessels, the diaphragm, and lymph nodes. The lymph node specimens from these control mice contained carcinoma in some samples and medullary mesenchymal cell hyperplasia in others. These results suggest that ILK expression leads to decreased growth and metastasis of tumor cells *in vivo*.

**B3d. ILK inhibits cell invasion *in vitro*:** To examine the effects of ILK on cell invasion, the ability of vector controls and ILK transfected MDA-MB-435 cells to degrade and invade vitronectin-coated polycarbonate membrane was investigated. As shown in Figure-7, cell invasion through membranes coated with vitronectin, was decreased by 60% in MDA-MB-435 cells expressing ILK compared to parental MDA-MB-435 cells.

**B3e. Regulation of  $\alpha 5\beta 1$  and  $\alpha v\beta 3$  integrins in the MDA-MB-435 breast cancer cell line:**

Cell adhesion and migration are controlled by the levels of integrins and by the amount of fibronectin matrix around the cell (19). Thus, a critical question is, does ILK regulate the expression of different integrins at the cell surface? To address this question, the expression levels of  $\alpha 5\beta 1$  and  $\alpha v\beta 3$  integrins were assessed in the ILK transfected and control MDA-MB-435 breast cancer cell lines by antibody-tagged FACS analysis. Monolayer cultures (60-80% confluency) ILK transfected and control cells were trypsinized and washed in culture medium. Cells ( $2.5 \times 10^5$ ) were pelleted and resuspended with integrin  $\alpha 5\beta 1$  monoclonal antibodies (MAB 1999, Chemicon Inc.),  $\alpha v\beta 3$  (MAB1976, monoclonal antibodies (Chemicon Inc.) or isotype control IgGs and incubated for 1h at  $4^\circ\text{C}$ . Cells were washed once in PBS/0.1% BSA and then incubated in PBS/0.1%BSA containing FITC-labeled goat anti-mouse IgG secondary antibody for 45 min. at  $4^\circ\text{C}$  protected from light. Cells were then washed and analyzed on a Becton -Dickinson FACScan flowcytometer. The results are shown in Figure-8. The ILK transfected cells expressed increased levels of the growth-suppressing integrin  $\alpha 5\beta 1$  (31% increase) and decreased levels of the growth-promoting integrin  $\alpha v\beta 3$  (22% decrease) compared to the

control cells. High  $\alpha 5\beta 1$  expression suppresses tumorigenicity in vivo (21) whereas perturbing the function of  $\alpha 5\beta 1$  with peptides that block its ligand binding suppresses experimental metastasis (21,22). The observation that ILK overexpression increases the levels of  $\alpha 5\beta 1$  is therefore very significant. ILK may function as a metastasis suppressor gene by modulating the levels of the growth-suppressing integrin  $\alpha 5\beta 1$  and the growth-promoting integrin  $\alpha v\beta 3$ .

### C. CONCLUSIONS:

Breast cancer is a major cause of morbidity and mortality in women in many parts of the world. It is estimated that over 46,000 women will die from breast cancer in the United States alone this year. Once breast cancer has been diagnosed, the most crucial question is whether the cancer is confined to the breast or whether it has already spread to distant sites. The reason for this concern is simple: metastasis, the spread of cells from the primary neoplasms to and growth at distant sites, is the most likely cause of death in breast cancer patients. Breast cancers vary widely in their clinical aggressiveness. Some cancers metastasize rapidly and kill the patient within a year of initial diagnosis, whereas others remain localized, never metastasizing during the lifetime of the patient. If truly localized, breast cancer can be cured by modified radical mastectomy. However, if the cancer only appears to be localized but in reality has metastasized, then systemic therapy is required. Unfortunately, there is no prognostic method to identify cells possessing the metastatic phenotype within a primary tumor population. In primary breast cancer, the axillary lymph node status is still the most important prognostic factor and is used for deciding on adjuvant therapy. However, the prognostic value of the axillary lymph node is not absolute, as 30% of node-negative patients still die within ten years because of recurrent disease and 30% of node-positive patients survive ten years without disease. Therefore, routine axillary lymph node dissection has recently become a matter of debate, and search for other factors to identify patients at high risk of (early) relapse is thus needed. It is hoped that the identification of biochemical or genetic alterations will provide markers that can be applied to these clinical problems.

Metastasis is a molecular event distinct from initial tumor formation and cells progress to metastatic capability after accumulating several genetic alterations. Loss of metastasis suppressor genes is an important event during progression of a tumor cell from a non-metastatic to a metastatic phenotype. Thus, knowledge of genetic loci and genes whose loss or inactivation contributes to metastasis development is of critical significance not only for basic knowledge, but also, perhaps, for the eventual design of novel therapeutic approaches and for crucial decisions of treatment and prognosis of the disease. We have reported that chromosome 11p15.5 exhibits loss of heterozygosity (LOH) in ~60% of breast tumors, and that there is a significant correlation between 11p LOH and clinical parameters which portend a more aggressive tumor and a more ominous outlook for the patient, such as aneuploidy, high S-phase fraction and the presence of metastasis in regional lymph nodes. Our data strongly suggest that chromosome 11p15.5 harbors a metastasis suppressor gene for human breast cancer. An intriguing candidate gene that we have mapped to the metastasis suppressor locus on chromosome 11p15.5 is Integrin-linked kinase (ILK). ILK is a newly identified serine/threonine kinase that binds to the cytoplasmic domains of both  $\beta 1$  and the  $\beta 3$  integrins and mediates the down-stream signaling events in integrin function. Recent evidence suggests that cell-cell and cell-matrix interactions, that are important prerequisites of the metastatic process, are modulated by integrins, and there is a growing body of evidence suggesting that variations in the expression of these molecules can have a profound effect on tumor biology. The role of Integrin-linked kinase in integrin-mediated signaling is not fully understood, however, the existing paradigm is that ILK may function as a protooncogene.



In preliminary experiments, we have provided evidence that ILK expression is down-regulated in primary breast tumors and in cell lines derived from metastatic breast tumors. We have shown that ILK overexpression inhibits the *in vitro* and *in vivo* growth of the highly metastatic breast cancer cell line MDA-MB-435. In addition, ILK overexpression stimulates the levels of the growth suppressing integrin  $\alpha 5\beta 1$  and inhibits the levels of  $\alpha v\beta 3$ , a growth promoting integrin. These innovative studies suggest a novel role for ILK in the etiology of breast cancer.

#### **D. KEY RESEARCH ACCOMPLISHMENTS:**

- Chromosome 11 harbors a breast cancer metastasis suppressor gene
- Integrin linked kinase (ILK) is a key candidate gene that maps to this region
- ILK expression is downregulated in breast carcinomas that metastasize
- ILK expression inhibits the *in vitro* and *in vivo* growth of the metastatic breast cancer cell line MDA-MB-435

These data suggest that ILK functions as a metastasis suppressor gene in breast cancer

#### **E. REPORTABLE OUTCOMES:**

- We have transfected the ILK gene into the metastatic breast cancer cell line MDA-MB-435 and have isolated four different clones that express different levels of ILK mRNA and protein. Using a nude mouse metastatic model, we have shown that ILK functions as a metastasis suppressor gene in breast cancer.
- These results are being prepared as a manuscript for publication.

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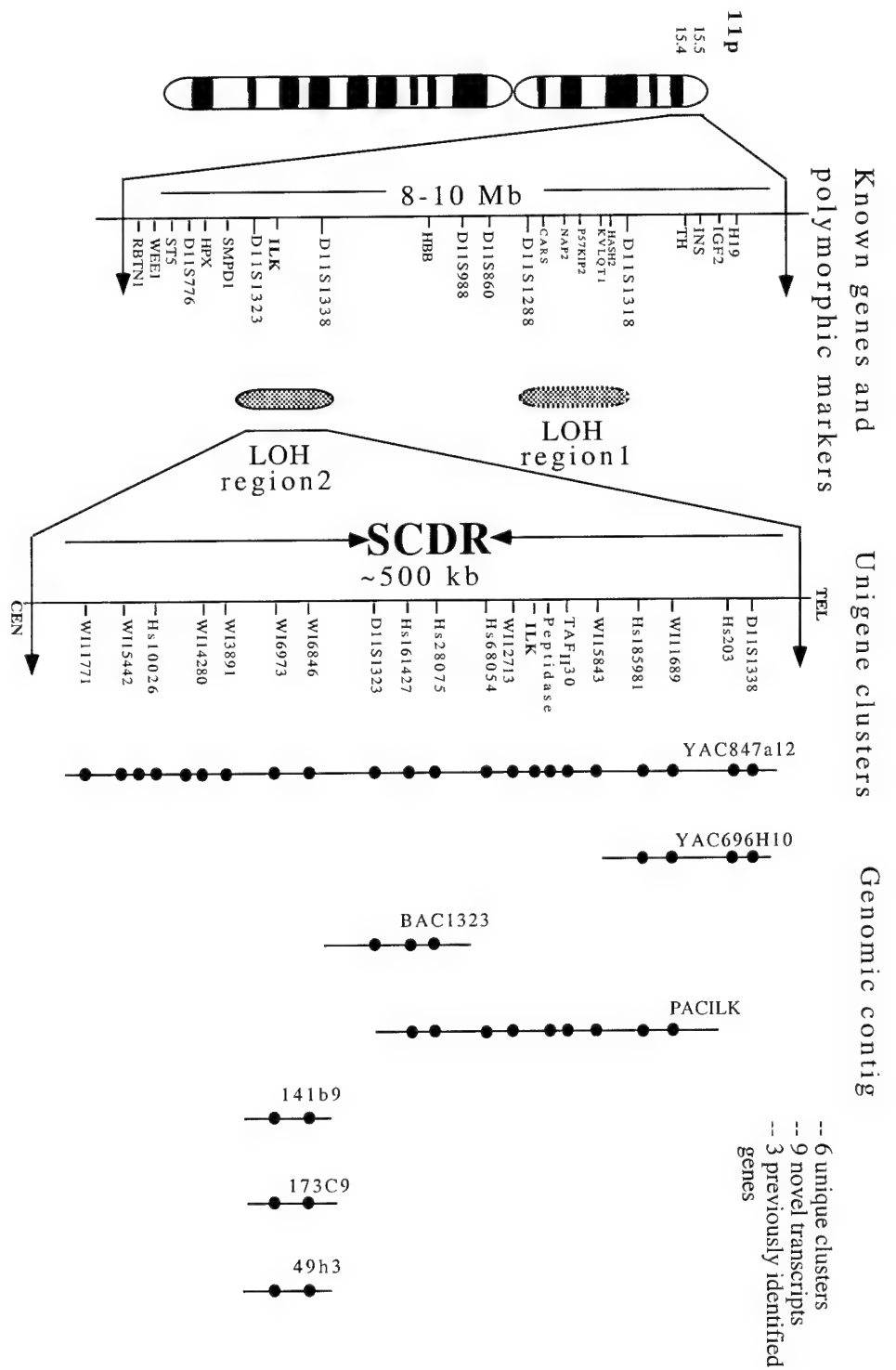


Figure 1. Schematic representation of chromosome 11p15.5 LOH regions 1 and 2. Genomic contig and transcript map of the metastasis suppressor locus (LOH region 2).

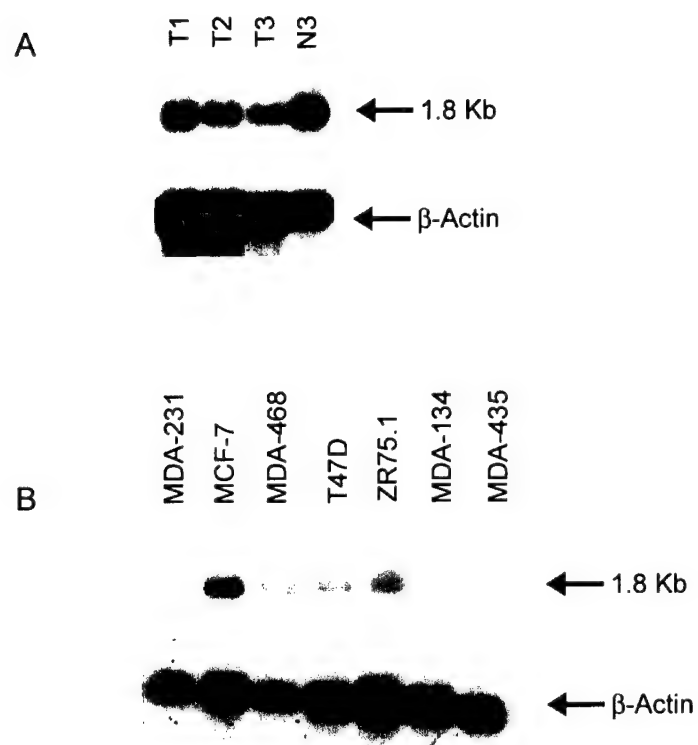


Figure 2. mRNA expression of Integrin-linked kinase. ILK cDNA clone was used to screen Northern blots from (A) Normal breast tissue (N3) and primary breast tumors (T1, T2, T3) and (B) Breast cancer cell lines. Hybridization with B-actin probe serves as controls to demonstrate mRNA loading.

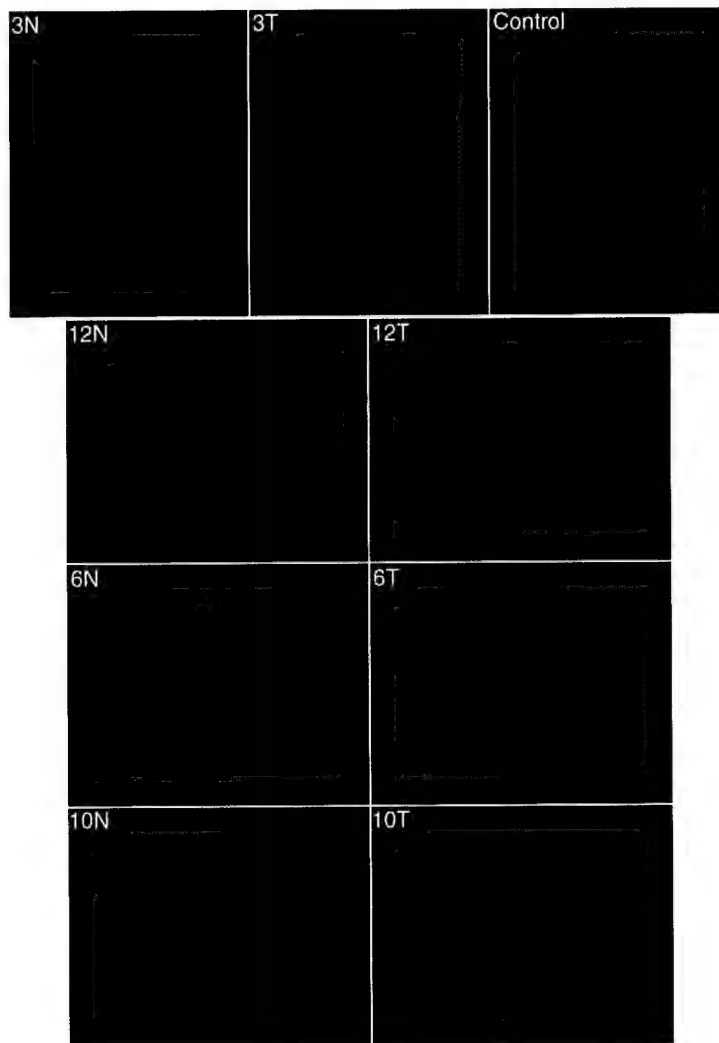
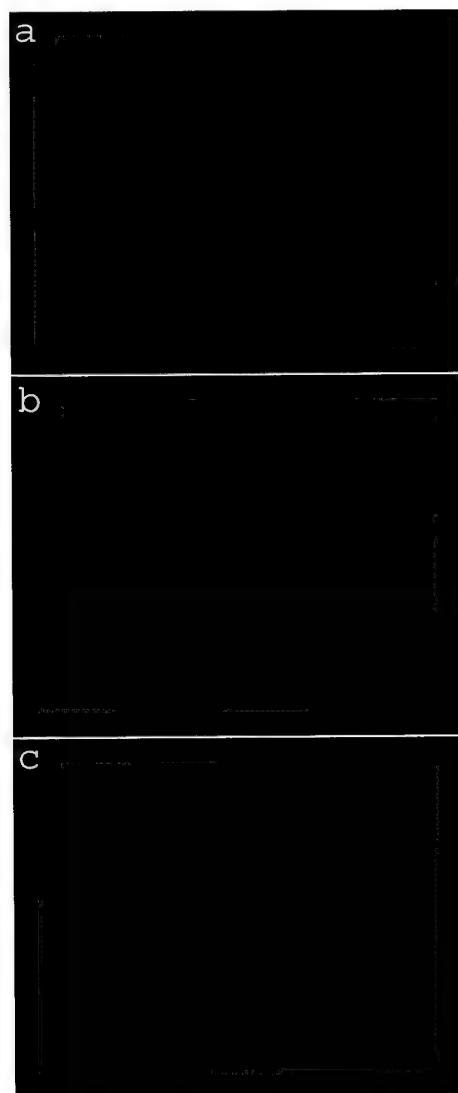


Figure 3. Immunohistochemical detection of Integrin Linked Kinase expression in normal breast ducts and in cancerous breast tissue.



Figure 4. Immunohistochemical analysis of ILK in the MDA-MB-435 cell line (b) before and (c) after transfection. (a) No primary antibody control.



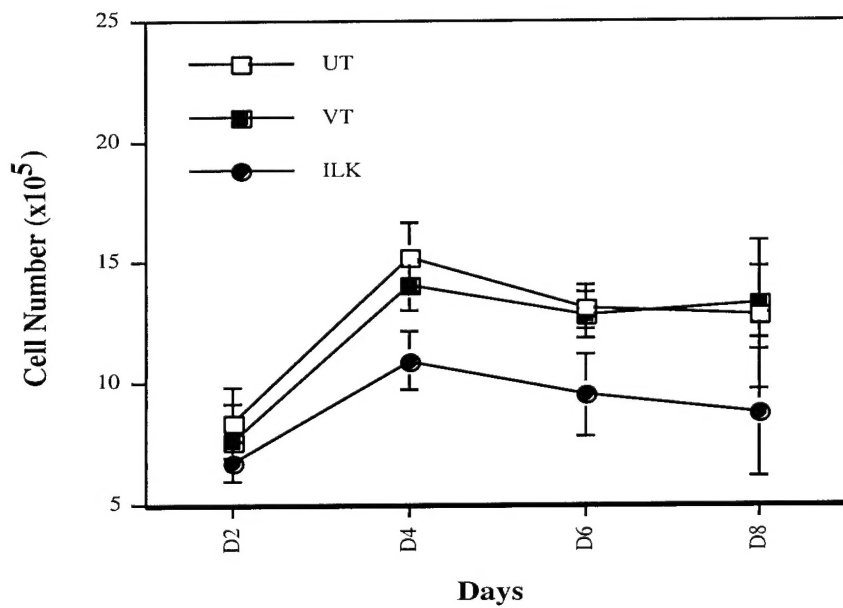
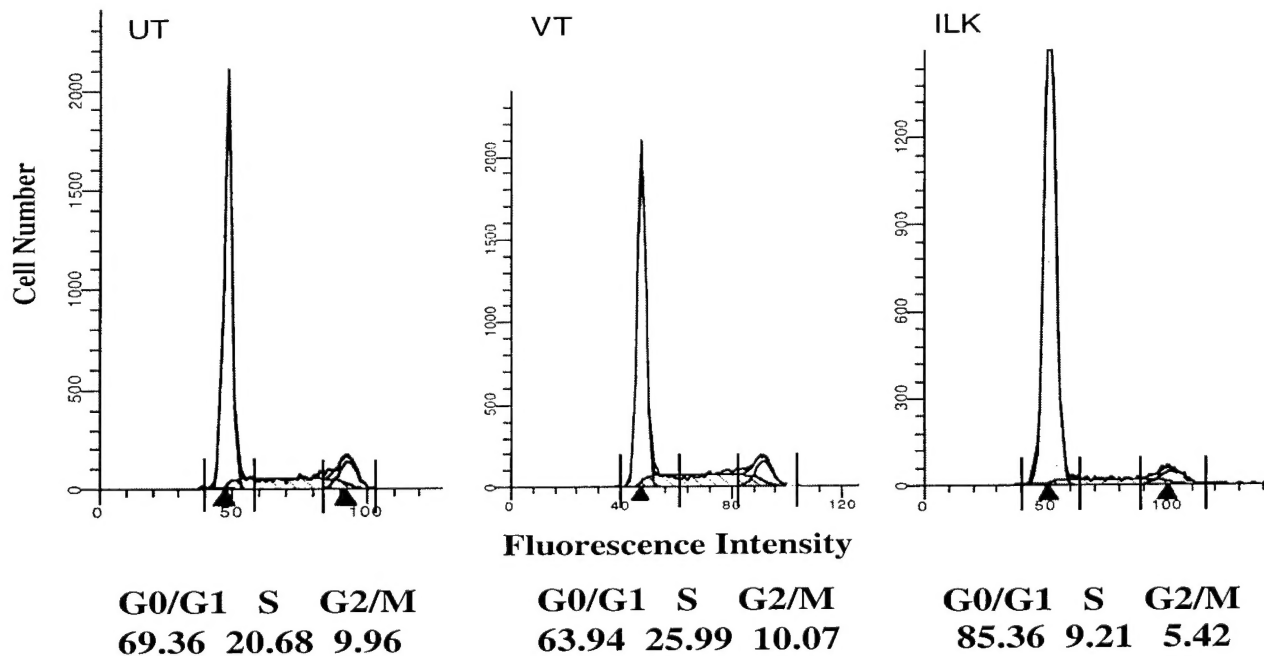


Figure 5. Growth inhibition by ILK. The human breast cancer cell line MDM-MB-435 was transfected with ILK cDNA or eukaryotic expression vector. 5a. Growth rates of ILK-transfected cells (●), as compared with vector transfected (■) and untransfected (□) cells. 5b. Cell cycle analysis in MDA-MB-435 cells (UT), transfected with vector (VT) and with ILK. The regions between the vertical lines from left to right represent cells in G0/G1, S and G2/M, respectively. Results are representative of three independent experiments.



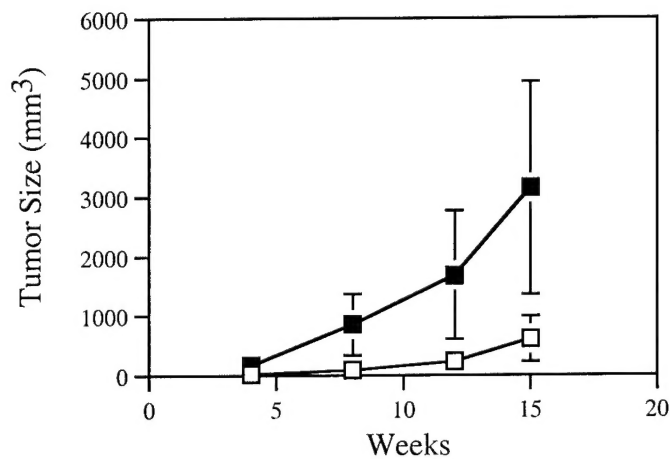


Figure 6. a. In vivo tumor growth of ILK transfected (■), and parental MDA-MB-435 cells in mammary fat pads of nude mice. Each point represents the mean  $\pm$  S.E of tumors.

b. Five  $\times 10^5$  cells of vector or ILK-transfected MDA-MB-435 cells were injected S.C into the mammary fat pad area below the nipple. Tumors were allowed to grow for 15 weeks at which time the mice were photographed and killed.



ILK transfected cells



Vector transfected cells

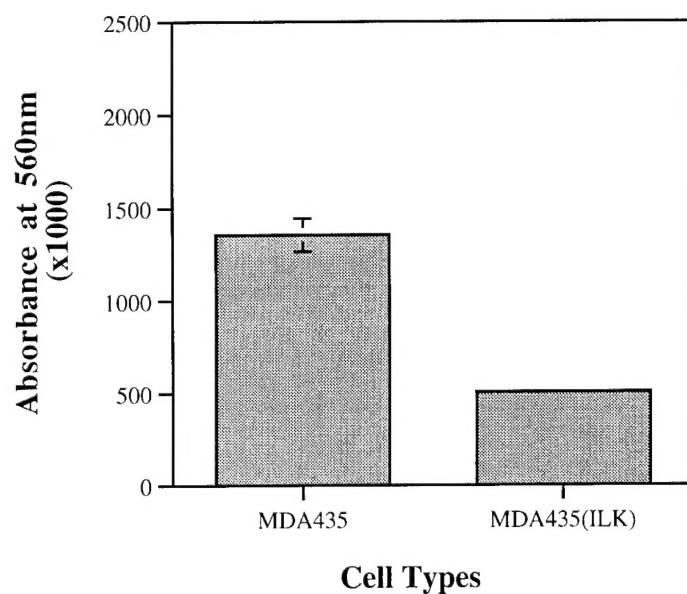
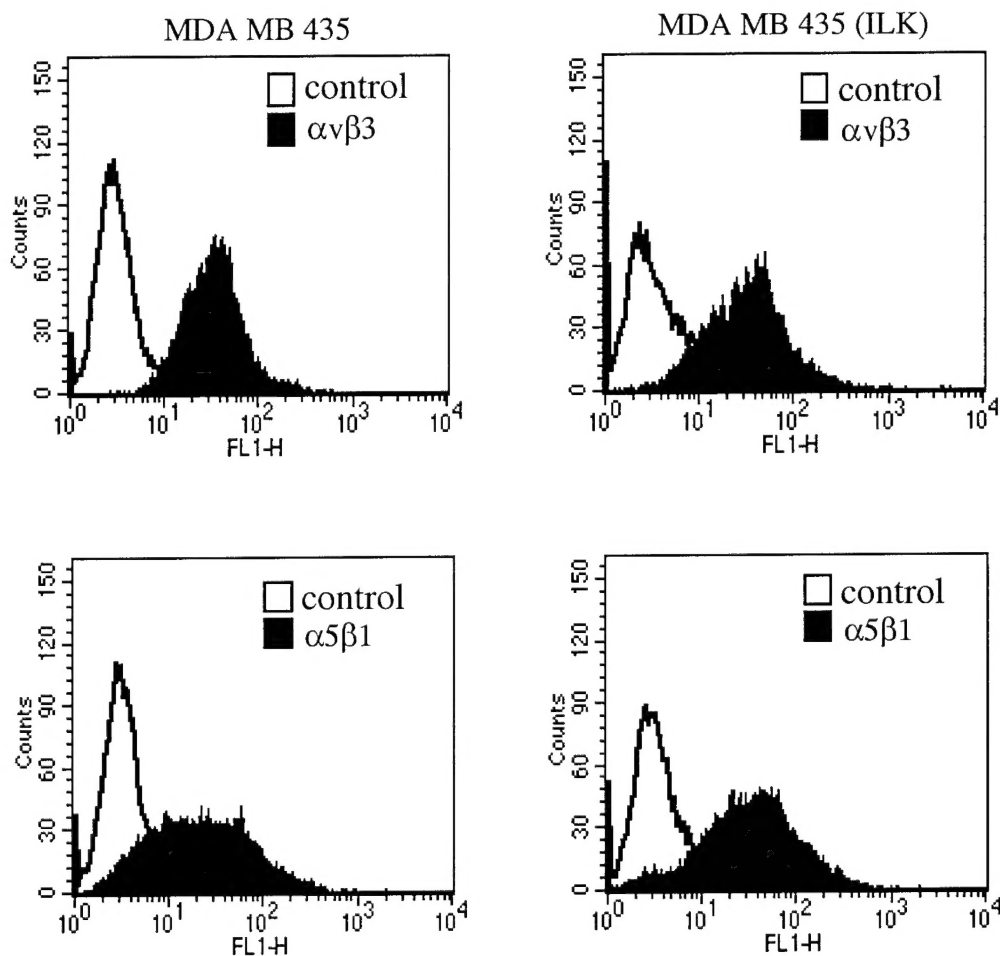


Figure 7. Cell invasion assay in parental MDA-MB-435 and ILK transfected cells. Cell invasion through vitronectin was analyzed using a modified Boyden chamber. Cells that invaded to the lower surface of the membrane were lysed and absorbance determined at 560nm.

A



B

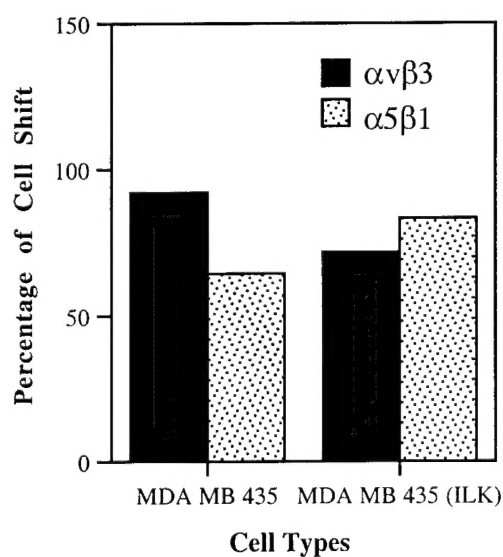


Figure 8. Flow cytometric analysis of  $\alpha 5 \beta 1$  and  $\alpha v \beta 3$  integrins expressed on the surface of ILK transfected and control cells. (A) The relative fluorescence intensity of cells stained with MAB1976 ( $\alpha v \beta 3$ ) or MAB1999 ( $\alpha 5 \beta 1$ ) integrin antibodies. X-axis, intensity of fluorescence; Y-axis, percentage of cell shift obtained for each integrin. (B) The percentage of cell shift with  $\alpha v \beta 3$  and  $\alpha 5 \beta 1$  antibodies in the MDA MB 435 control and ILK transfected cells. Each graph is a representative example of an experiment that has been repeated at least three times.